4. A Deuterium-Labeling Technique to Study *myo*-Inositol Metabolism

INTRODUCTION

myo-Inositol is widespread in nature, and its roles in plants have been reviewed recently (Loewus and Dickinson, 1982; Loewus and Loewus, 1980, 1983; see also Chapters 1–3, this volume). Although it is a component of membrane lipids, *myo*-inositol has an especially important role in plants as a precursor of uronic acid and pentose residues of cell wall polysaccharides (Loewus and Dickinson 1982; Loewus and Loewus, 1980, 1983). *myo*-Inositol is a major carbon source for cell wall synthesis in growing tissues of monocots and dicots (Sasaki and Loewus, 1980, 1982; Sasaki and Taylor, 1984, 1986) and is therefore essential for plant growth. This chapter describes new tools for studying the roles of *myo*-inositol in plant metabolism.

SYNTHESIS AND PURIFICATION OF DEUTERATED INOSITOL

Although radioisotope-labeled *myo*-inositol (^{3}H or ^{14}C) has been widely used for studies of *myo*-inositol metabolism, labeling with a stable isotope has several advantages over the radioisotope methodology: 1) Stable isotopes are not radioactive and are therefore safer and easier to handle; 2) labeled and nonlabeled compounds are simultaneously analyzed with the aid of gas chromatography-mass spectrometry (GC-MS) or liquid chromatography (LC)-MS ("one-pot analysis"); 3) redistribution of stable isotope atoms can be readily detected by selected ion

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monitoring; 4) specific labeling of biological compounds with a stable isotope allows use of either ^{2}H or ^{13}C nuclear magnetic resonance (NMR) for noninvasive investigations of biological systems.

Raney Nickel-Catalyzed Deuterium-labeling

The carbon-bound proton atoms of carbohydrates are easily exchanged with ^{2}H in deuterium oxide in the presence of Raney nickel as a catalyst (Koch and Stuart, 1977). However, extensive isomerization and other reactions occur during the Raney nickel-catalyzed ^{1}H-^{2}H exchange reaction of *myo*-inositol (Sasaki et al., 1987). Seven inositol isomers were detected and identified in the exchange reaction products by GC-MS using a capillary column of SE-30 or SE-54. The presence of many unknown peaks in the reaction products and the disappearance of the inositol peaks after 24 hours of boiling indicate that degradation of the inositols as well as their isomerization occurs during the ^{1}H-^{2}H exchange reaction. The isomerization and degradation reactions constitute a serious hindrance to both the preparation and purification of deuterium-labeled *myo*-inositol using Raney nickel as a catalyst.

Purification of Deuterated Inositol Isomers

Anion-exchange chromatography with Dowex 1 (borate form) resin (0.9 × 60 cm) using a combination of gradient and stepwise elution with increasing concentration of boric acid provides complete separation of *myo-, scyllo-, allo- and muco-inositols, and partial separation of neo-* from *chiro*-inositol as their borate complexes (Sasaki et al., 1987). The column can be used repeatedly after washing with water. Because boric acid is easily removed as methyl borate ester by repeated evaporation with methanol, it is preferred over potassium or sodium borate solution, which are commonly used for chromatography and electrophoresis of neutral sugars. This eliminates the need for cation-exchange chromatography for the removal of Na^{+} or K^{+} ions.

neo-Inositol and *chiro*-inositol can be separated by recrystallization because *chiro*-inositol is much more soluble in water than *neo*-inositol. Most of the *chiro*-inositol remains in the soluble fraction even after four crystallizations, whereas *neo*-inositol is completely removed from the fraction. Plants (especially leguminous plants) also contain *chiro*-inositol in free and methylated forms; however, their roles and metabolic fate in plants are not fully understood (Loewus and Dickinson, 1982; Loewus and Loewus, 1980). Thus, the deuterium-labeled *chiro*-inositol, prepared and purified by the method outlined above, will be useful for studies of the roles and metabolism of this inositol isomer.

METABOLISM OF *myo*-INOSITOL IN GERMINATING BEANS (*Phaseolus vulgaris* L.)

^{3}H Distribution in Seedling Fractions

The per-C-deuterated *myo*-inositol prepared and purified by the method outlined above was successfully used for the study of *myo*-inositol metabolism in germinating beans (Sasaki et al., 1989). For this study, it was assumed that there was no discrimination between non-deuterated (but tritiated) and per-C-deuterated *myo*-inositol. A 1 ml of per-C-deuterated *myo*-inositol solution (0.2 M) containing a small amount of *myo*-[2-^{3}H]inositol (to provide a radiolabeled marker) was supplied to each of five bean seeds by imbibition under sterile conditions as reported elsewhere (Sasaki and Taylor, 1984, 1986). Germinating seedlings were kept under sterile conditions and harvested after 3 days, when the hypocotyl length had reached 1 cm. Approximately 9.5% of ^{3}H was taken up by the germinating seedlings after 72 hours. Each of the seedling parts (cotyledons, hypocotyl, and root) was ground in 80% ethanol and extracted with 80% ethanol and then with chloroform-methanol (1:1/v:v). Starch in the insoluble residue was removed by a treatment with α-amylase (from porcine pancreas). The starch-free cell wall residue was first hydrolyzed in 2N trifluoracetic acid (TFA). The insoluble, cellulosic fraction was further hydrolyzed in 72% H_2SO_4 (Sasaki and Taylor 1984).

The majority of ^{3}H in each seedling part was found in the 80% ethanol-soluble fraction. Virtually no ^{3}H was removed from the ethanol-insoluble residue with chloroform-methanol. In the growing hypocotyl and root, 15 to 19% of ^{3}H remained in the starch-free cell wall residue, and about 90% of this remaining ^{3}H was released from the cell wall by TFA hydrolysis.

^{2}H Distribution in Cell Wall Sugar Residues

Because most of ^{3}H in the cell wall was released by 2N TFA hydrolysis, ^{2}H distributions in the TFA-soluble sugars were further investigated by GC-MS analysis after conversion of these sugars to their alditol acetates (Sasaki et al., 1989). A fragment ion, m/z 187, was chosen as a characteristic ion of nonlabeled sugars and m/z 191 for ^{2}H-labeled neutral

sugars. Total sugar compositions were obtained from the total ion chromatograms. As seen in Figure 1, seven neutral sugars with m/z 187 were detected in the TFA-hydrolysate of hypocotyl cell walls. However, only arabinose and xylose residues were labeled with ^2H. No fragment ion with m/z 191 was found in other neutral sugar residues. The same results were obtained from root cell walls. Fragment ions with m/z 191 were found only in arabinose and xylose residues (Fig. 2). On the other hand, none of these seven neutral sugar residues were labeled with ^2H in cotyledon cell walls, indicating that new cell wall synthesis from *myo*-inositol did not occur in this storage organ during germination (Fig. 3).

Galacturonic acid and glucuronic acid were found as major acidic sugar residues in the bean cell walls. However, selected ion chromatograms of the acidic sugar residues clearly showed that only galacturonic acid residues of hypocotyl and root cell walls were labeled with ^2H. Glucuronic acid may not be required for cell wall synthesis during very early stages of germination. Alternatively, UDP-glucuronic acid within

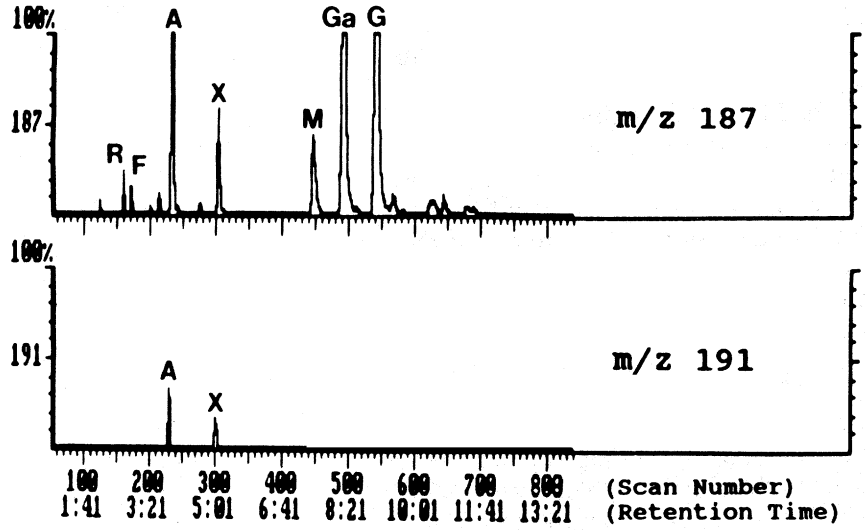

Fig. 1. Selected ion chromatograms of neutral sugar residues of bean hypocotyl cell walls. Cell walls were hydrolyzed with 2N-TFA, and the released neutral sugars were converted to their alditol acetates. Alditol acetates were assayed by GC-MS, equipped with a capillary column of SP-2330 (15 m × 0.25 mm), using selected ion monitoring of m/z 187 for nonlabeled sugars and m/z 191 for ^2H-labeled sugars. The numbers on the axis of ordinates (187, 191) show fragment ions with m/z 187 and m/z 191, respectively. The numbers on the axis of abscissas are scan numbers (upper) and retention times in minutes (lower). R, rhamnose; F, fucose; A, arabinose; X, xylose; M, mannose; Ga, galactose; G, glucose.

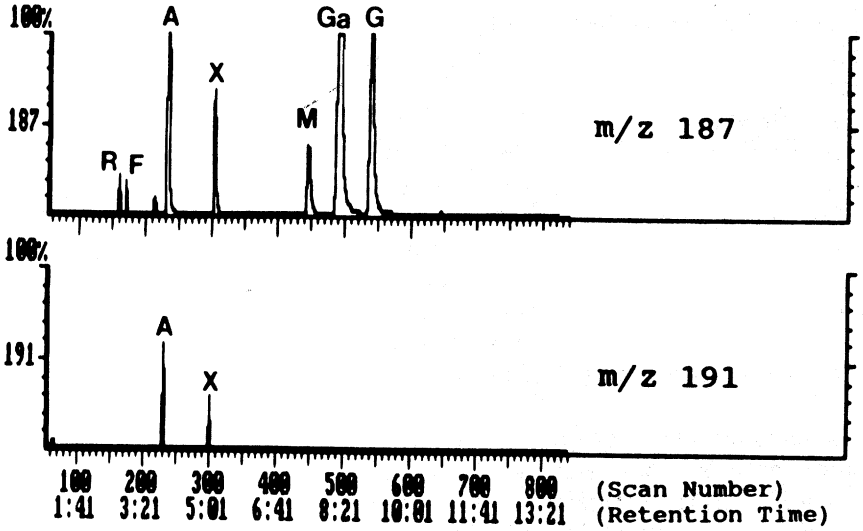

Fig. 2. Selected ion chromatograms of neutral sugar residues of bean root cell walls. Analytical conditions and symbols are as in Figure 1.

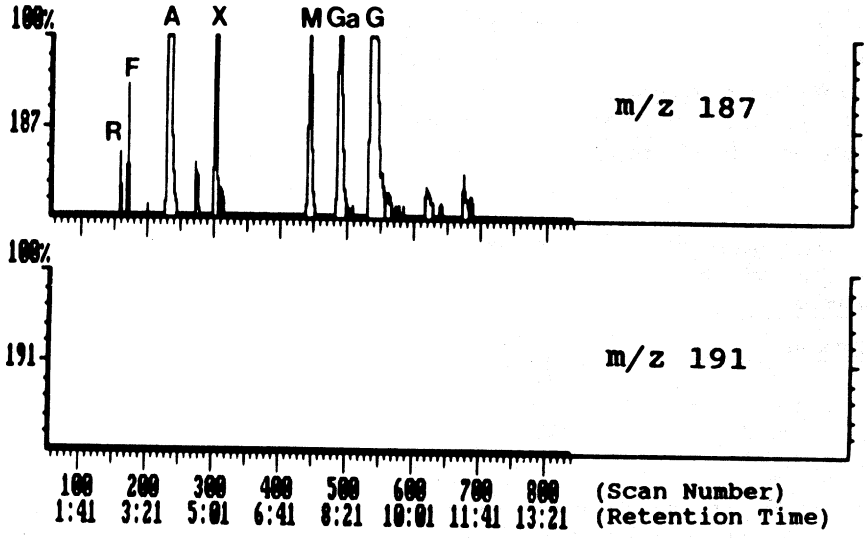

Fig. 3. Selected ion chromatograms of neutral sugar residues of bean cotyledon cell walls. Analytical conditions and symbols are as in Figure 1.

the *myo*-inositol oxidation pathway may be unavailable for biosynthetic processes of cell walls involving the transfer of glucuronosyl groups during this period of germination growth. In cotyledons, none of these acidic sugars were labeled (data not shown).

Selected ion chromatograms also showed that none of the fragment ions with m/z 190 or 189, which would be indicative of only three or two ^2H per fragment, were produced from any neutral sugar residues. Moreover, no ion with m/z 189, which would be indicative of two ^2H per fragment, was found in either galacturonic acid or glucuronic acid residues. The results clearly show that all deuterated sugar residues retained five ^2H atoms and that no ^2H redistribution occurred during the conversion of ^2H-labeled *myo*-inositol into arabinose, xylose, and galacturonic acid residues.

These results indicate that ^2H-labeled *myo*-inositol was metabolized only via the *myo*-inositol oxidation pathway to provide these sugar residues for the synthesis of cell wall polysaccharides and that glucogenesis from *myo*-inositol (Rosenfield et al., 1978) does not occur in germinating beans.

STOICHIOMETRIC CONSIDERATION OF THE *myo*-INOSITOL OXIDATION PATHWAY

Comparison of the amounts of deuterated and nondeuterated arabinose, xylose, and galacturonic acid residues provides quantitative information on the amount of per-C-deuterated *myo*-inositol that was used for the synthesis of these cell wall sugar residues during 3 days of imbibition treatment (Table I). More than 20% of xylose and galacturonic acid residues and about 10% of arabinose residues in the hypo-

TABLE I. Comparison of Deuterated and Nondeuterated Sugars in the Cell Walls of 3-Day-Old Bean Seedlings as Determined From Alditol Acetate GC-MS Chromatograms Using Selected Ion Monitoring*

	Hypocotyl		Root	
	^2H	^1H	^2H	^1H
Arabinose	9.8	90.2	18.1	81.9
Xylose	22.9	77.1	27.3	72.7
Galacturonic acid	20.2	79.8	29.2	70.8

* Expressed in mole %: Percentages of deuterated and nondeuterated sugar residues in the total amount of each sugar.
^2H = deuterated, ^1H = nondeuterated.

cotyl cell wall were labeled with ^{2}H. In roots, nearly 30% of xylose and galacturonic acid and 20% of arabinose residues were deuterated.

Per-C-deuterated *myo*-inositol supplied externally to bean seeds probably followed the metabolic fate of endogenous sources of *myo*-inositol and was converted into galacturonic acid, xylose, and arabinose via the *myo*-inositol oxidation pathway for cell wall synthesis. There are two endogenous sources of *myo*-inositol in seeds: stored *myo*-inositol (as a free form, galactinol and phytic acid) (Sasaki and Loewus, 1980, 1982), and de novo synthesis of *myo*-inositol from sugar reserves (Sasaki and Taylor, 1986). In germinating wheat, *myo*-inositol stored as a free form and galactinol are rapidly used for cell wall synthesis. Phytic acid, on the other hand, is metabolized slowly (Sasaki and Loewus, 1982). In beans, phytic acid hydrolysis also starts slowly, 3 days after germination (Gibbins and Norris, 1963), and the released *myo*-inositol is used for cell wall synthesis at actively elongating and nonelongating regions (Sasaki and Taylor, 1984). De novo synthesis of *myo*-inositol occurs during very early stages of germination (within 72 hours of imbibition) (Sasaki and Taylor, 1986).

Considering these endogenous sources of nondeuterated *myo*-inositol in bean seeds and the nondeuterated pentose and galacturonic acid residues that were present in cell walls of the embryonic axis prior to imbibition, it is remarkable that such a significant portion of the cell wall pentose and galacturonic acid residues was deuterated in only 3 days of imbibition treatment (Table I). This indicates that the *myo*-inositol oxidation pathway is the major pathway providing these sugar residues for cell wall synthesis during germination of beans.

Thus, the combination of the stable isotope labeling and GC-MS analyses simultaneously provides ^{2}H-labeled, nonlabeled, and total sugar compositions. The selected ion monitoring provides additional information on the distribution of ^{2}H in each sugar molecule. Analysis of selectively labeled cell wall polysaccharides with ^{2}H is in progress using a noninvasive method (^{2}H-NMR). Clearly, the use of per-C-deuterated *myo*-inositol and GC-MS analysis will provide a powerful technique for future studies of *myo*-inositol metabolism.

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